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The Release of High-Molecular-Weight Alkaline Phosphatase and Leucine Aminopeptidase into the Media of Cultured Human Cells[1]

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SUMMARY

Using exclusion from Sepharose 4B as our criterion, we have found a high-molecular-weight form of alkaline phosphatase and of leucine aminopeptidase which are released into the culture media by the FL amnion cell line. A low-molecular-weight form of leucine aminopeptidase is also found to contribute to the total levels of this enzyme in the media.

The levels of these enzymes increased during the growth cycle of the culture, paralleling the increase in cell density, suggesting that the two events may be related.

This phenomen in culture suggests a possible explanation for the appearance of similar enzyme forms in patient serum and fluids originating from diseased tissue.

INTRODUCTION

High-molecular-weight enzyme forms have been discovered in the serum of patients with a variety of neoplastic and nonneoplastic disorders (1 3, 6). Although the significance of this phenomenon remains obscure, it has been speculated that these enzymes are released from the affected organ into the blood stream as a complex with the properties of a membrane fragment.

We have observed a high-molecular-weight species of alkaline phosphatase and leucine aminopeptidase in the media of a cultured human cell line (FL amnion), a hitherto unreported phenomenon.

In this report, we shall describe these enzymes with regard to exclusion or nonexclusion from Sepharose 4B in the media of cultured cells, and we shall demonstrate some similarities with those found in serum and fluid specimens of an ovarian cancer patient. Other evidence suggests that the appearance of these high-molcular-weight enzyme forms is related to the stage of growth of the culture.

MATERIALS AND METHODS

Culture Method. Cells were grown in Eagle's minimum essential medium (Grand Island Biological Company, Grand Island, N. Y., Catalog 143EG). Medium was supplemented with calf serum at a final concentration of 10%, penicillin (100 units/ml), streptomycin (100 μg/ml), Fungizone (2.5 μg/ml), and sodium bicarbonate at a final concentration of 0.1%.

Cells. FL amnion cells were purchased from the American Type Culture Collection, Rockville, Md. (Catalog CCl 62).

Alkaline Phosphatase Assay. Alkaline phosphatase activity was measured using disodium phenylphosphate substrate at pH 10.7 as previously described (4).

Leucine Aminopeptidase Assay. With a method similar to that described by Rockerbie and Rasmussen (5), samples were assayed for leucine aminopeptidase. Phosphate-buffered (pH 7.2) substrate (L-leucyl β-naphthylamide) was mixed with samples and prewarmed to 37° in a circulating water bath. An aliquot of this mixture was pipetted into a thin-walled test tube (75 × 7 mm) and read on a Turner fluorometer. Enzyme activity was expressed as nmoles β-naphthylamine released per min per ml. The standard curve was obtained by diluting a leucine aminopeptidase calibration standard solution (Sigma Chemical Co., St. Louis, Mo., Catalog 251-10).

Column Chromatography. Sepharose 4B columns (1.6 × 15.0 cm) (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), were used throughout this study. The elution buffer used was 0.05 M Tris-acetate, pH 8.0.

Separation of High-Molecular-Weight Alkaline Phosphatase and Leucine Aminopeptidase from Media. Falcon T-25 tissue culture flasks were inoculated with 500 × 10^3 FL amnion cells in 5 ml of media. The media from duplicate cultures were removed daily and analyzed for different molecular-weight forms of alkaline phosphatase and leucine aminopeptidase.

The media were poured off and centrifuged for 5 min at 1,000 rpm to sediment floating cells. The resulting supernatant solution was again centrifuged at 10,000 rpm for 10 min to remove cell debris, which otherwise tended to clog up the Sepharose 4B column.

Three ml of this last supernatant were layered on a Sepharose 4B column, and 1-ml fractions were collected. The excluded enzymes were observed in the void volume of the column (Tubes 7 to 14).

RESULTS

Chart 1 shows the levels of leucine aminopeptidase and alkaline phosphatase in the media as a function of time in

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culture. The levels of both enzymes increase during the growth cycle of the FL amnion cell line at roughly parallel rates. The leucine aminopeptidase shows a faster initial rise, while the level of alkaline phosphatase rises more rapidly later in the growth cycle.

Charts 2 and 3 demonstrate the Sepharose 4B elution profiles of alkaline phosphatase and leucine aminopeptidase in the media at various intervals during the growth cycle. In

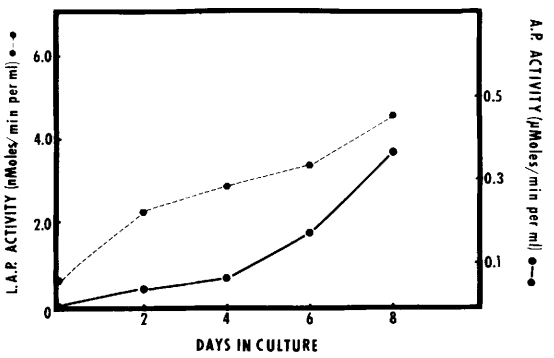

Chart 1. Total leucine aminopeptidase (*L.A.P.*) and alkaline phosphatase (*A.P.*) activity in the media during growth in culture. Alkaline phosphatase activity (●) is expressed as μmoles disodium phenylphosphate hydrolyzed per min per ml media. Leucine aminopeptidase activity (◆ is expressed as nmoles L-leucyl β-naphthylamide hydrolyzed per min per ml media.

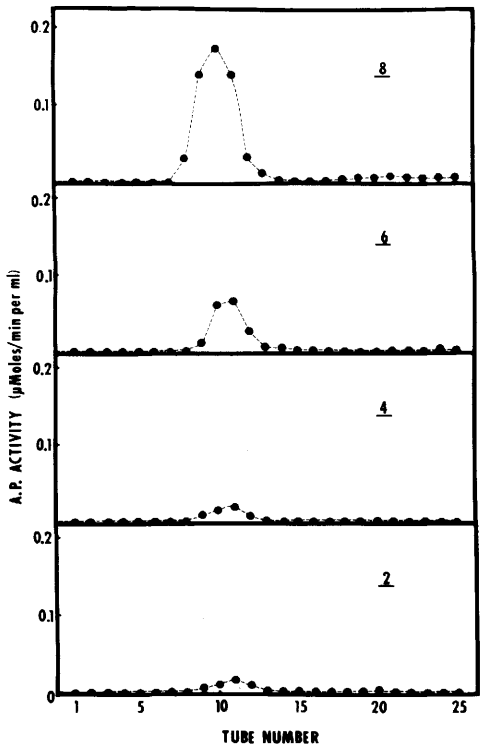

Chart 2. Sepharose 4B elution profile of alkaline phosphatase (*A.P.*) during growth in culture. Enzyme activity is expressed as μmoles substrate hydrolyzed per min per ml of each of the fractions indicated on the abscissa. Media were sampled at 2-day intervals, and each profile represents the media of different days in culture (*underlined numbers*). No elution profile is presented for the control medium because there was no detectable enzyme activity.

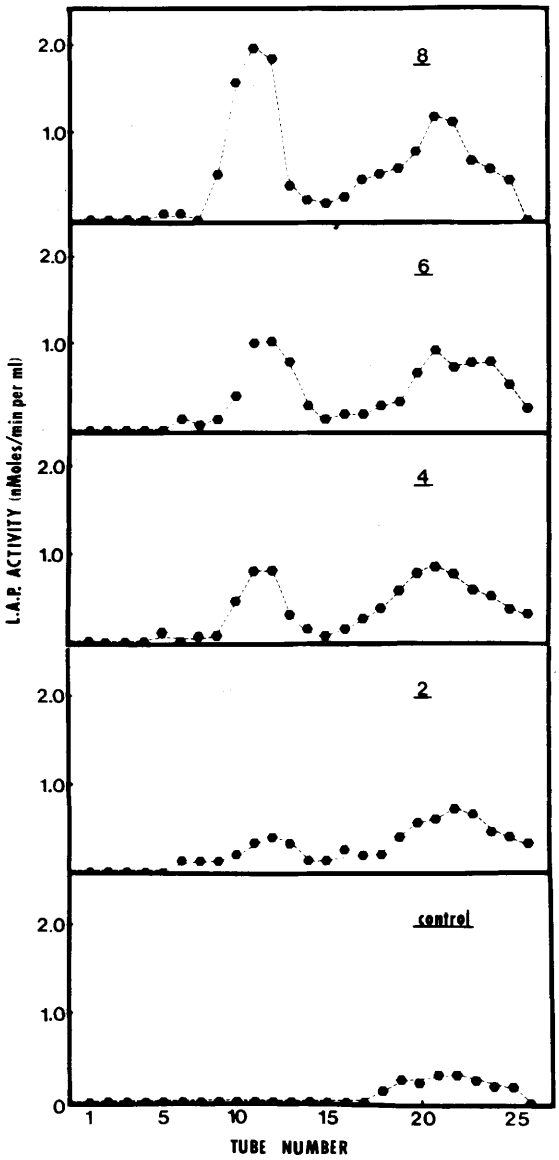

Chart 3. Sepharose 4B elution profile of luecine aminopeptidase (*L.A.P.*) in the media during the growth cycle. Activity is expressed as a rate of hydrolysis of L-leucyl β-naphthylamide per min per ml of each of the fractions indicated on the *abscissa*. The media were harvested at 2-day intervals (*underlined numbers*). The unused control medium graph is found to have enzyme activity in the retained lower-molecular-weight fractions, due to the calf serum.

Chart 2 it can be seen that all the alkaline phosphatase activity in the media is found in the heavy-molecular-weight-excluded fractions (Tubes 7 to 14). In this experiment, no low-molecular-weight alkaline phosphatase was detectable in the control media or in media harvested during the growth cycle. Thus, it appears that the increase in released alkaline phosphatase in the media during growth in culture is due exclusively to a heavy-molecular-weight form.

In some experiments, we have observed the presence of a low-molecular-weight form of alkaline phosphatase in the unused control media which is attributed to the calf serum.

With respect to leucine aminopeptidase, Chart 3 depicts the Sepharose 4B elution profile of this enzyme in the media

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at different stages of the growth cycle. The control unused medium is found to contain only a low-molecular-weight form of the enzyme, which is due to the calf serum. Media that were used to support cell growth exhibit increasing amounts of both low- and high-molecular-weight leucine aminopeptidase activity. The increase in the high-molecular-weight leucine aminopeptidase closely parallels the elevation in high-molecular-weight alkaline phosphatase shown in Chart 2. The rise in the low-molecular-weight leucine aminopeptidase is less pronounced but greater than the control levels of leucine aminopeptidase.

DISCUSSION

We have shown that alkaline phosphatase and leucine aminopeptidase are released in progressively greater amounts into the culture media in a heavy-molecular-weight form during the growth cycle, as indicated by its exclusion from the Sepharose 4B gel matrix.

The characteristics of these enzymes are similar to those described by Dymling (3) in the serum of a patient with biliary occlusion, and which has since been shown to be present in ovarian cancer patient ascites fluids (1), serum of patients with malignant bone cancer, metastatic liver disease, and nonmalignant liver disease (2). Shinkai and Akedo (6) determined that a hepatic cancer patient serum contained a similar high-molecular-weight alkaline phosphatase which was shown to be a lipid protein complex exhibiting 5 other enzyme markers of the plasma membrane. Although the source of this enzyme is unknown, it possibly represents a plasma membrane fraction from the cells of the diseased organ.

Additionally, it was determined that the increase in leucine aminopeptidase in the media is due to the release of at least 2 molecular-weight species of this enzyme. One has the same Sepharose 4B exclusion pattern resembling heavy-molecular-weight alkaline phosphatase and another low-molecular-weight form which also increases somewhat during the growth cycle.

From these results, it is reasonable to suggest that perhaps the progressive increase in the appearance of a heavy-molecular-weight alkaline phosphatase in the ascites fluids of ovarian cancer patients (1) is a reflection of increased cell density in the peritoneal cavity which occurs during the course of the disease.

We are studying the phenomenon of overgrowth in cultured cells, as well as in tumor-bearing animals, to determine the mechanism that releases heavy-molecular-weight forms of cellular enzymes and subsequently to establish the diagnostic potential and biological significance of this enzyme complex in neoplasia.

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